ENZYMATIC ACTIVITY AND MYCOTOXIN-PRODUCING POTENTIAL OF FUNGI ISOLATED FROM ROTTED LEMONS

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ABSTRACT - Nine species related to seven genera were recovered from rotting lemon (*Citrus limon* Syn.) fruits. *Alternaria alternata* appeared to be the main causer of lemon rot followed by *Aspergillus flavus* and *A. niger*. From the isolated fungi, 37 isolates were screened for their ability to produce some enzymes on solid media. Data clearly showed that not all the tested isolates were only able to produce cellulase but most of them were also good producers of this enzyme.In addition to cellulase, pectin lyase (PL) and poly-galacturonase (PG) production were also verified by many of these isolates. During screening for mycotoxigenicity of the isolated fungi on liquid medium, 17 out of 43 isolates were toxigenic. *Alternaria alternata* produced alternariol (AOH) and alternariol monomethyl ether (AME) and *A. flavus* produced aflatoxins B_1 and B_2. *Alternaria* toxins and aflatoxins B_1 and B_2 were also produced in healthy lemon fruits infected with toxigenic isolates of *Alternaria alternata* and *A. flavus*, respectively. The presence of these mycotoxins in infected lemons may constitute a potential hazard to human health.

RÉSUMÉ - Neuf espèces fongiques appartenant à sept genres ont été isolées de citrons moisis (*Citrus limon* Syn.). *Alternaria alternata* apparaît être le principal agent responsable du pourrissement des citrons, suivi de *Aspergillus flavus* et *A. niger*. 37 isolats ont été testés afin de déterminer leur aptitude à produire des enzymes sur milieu solide. Les résultats montrent clairement que non seulement la totalité des isolats produisaient des cellulases, mais étaient en plus de bons producteurs. En plus des cellulases, pour de nombreux isolats les productions de pectine lyase (PL) et de polygalacturosase (PG) ont pu être mises en évidence. 17 des 43 isolats se sont également montrés producteurs de mycotoxines en milieu liquide. *Alternaria alternata* produit de l'alternariol monomethyl ether (AME) et *Aspergillus flavus* produit des aflatoxines B_1 et B_2. Les toxines de l'*Alternaria* et les aflatoxines B_1 et B_2 étaient également produites sur citrons sains respectivement pour *A. alternata* ■ *A. flavus*. La mise en évidence de ces mycotoxines pourrait indiquer l'existence d'un risque potentiel pour la santé humaine résultant de la consommation de citrons.

INTRODUCTION

Fungal spores are usually present on many important fruits and vegetables which may become visibly infected, particularly after the tissues are weakened. Extensive deterioration results in huge economic loss.

Aspergillus spp., *Fusarium* sp. and *Rhizopus oryzae* were isolated from oranges (Fawole & Odunfa, 1992). Different *Penicillium* species were recorded as a cause of decay of citrus fruits (Kursanoff & Alexeyeva, 1938; Wei, 1940). Citrus fruits are also known to be affected by *Alternaria* rot (Singh, 1980; Stinson et al., 1981).

The relative ease with these fungi carry out the pathogenicity is directly linked to their capability to produce some extracellular enzymes. The role of cell-wall degrading enzymes during pathogenesis is well documented (Albersheim et al., 1969; Mehta et al., 1974).

In addition to deteriorated fruits, some toxic fungal metabolites may be produced. It has been documented (Wildman et al., 1967), that a number of fruits and fruit juices support aflatoxin production in appreciable quantity. The experimental production of aflatoxins in citrus fruits have been reported by different workers (Singh & Sinha, 1982; Varma, 1985). Different mycotoxins were produced in fungi- infected oranges and lemons (Stinsons et al., 1981).

The physiological studies on fungi causing lemon rot in Egypt are scarce. Hence, this work was designed to study the mycoflora which cause this disease. In addition, enzymatic activity and mycotoxin-producing potential of these fungi were also assessed.

MATERIALS AND METHODS

Source of samples. A total of 50 samples of lemon (*Citrus limon* Syn.) with surface lesions from fungal infection were collected from lemon fields, Faculty of Agriculture, Assiut University, Assiut, Egypt.

Isolation and identification of moulds. Tissue fragments were excised from lesions of infected fruits and were plated on Czapek's dextrose agar medium supplemented with rose bengal (65 ppm) as abacteriostatic agent (Smith & Dawson, 1944). Inoculated plates were incubated for 7-10 1days at 28°C. The resulting moulds were isolated and identified.

Enzymatic activity of the isolated fungi. Thirty seven fungal isolates from fungi recovered during this investigation were screened for their ability to produce some enzymes on solid media. These fungi were *Acremonium strictum, Alternaria alternata, Aspergillus flavus, A. fumigatus, A. niger, Cochliobolus spicifer, Mucor hiemalis, Penicillium italicum* and *Ulocladium atrum.*

Cellulase activity was studied by using the method described by Eggins & Pugh (1962). The tested fungi were grown on medium contained (gm/L), Ammonium sulphate, 0.5; L-asparagine, 0.5; Potassium dihydrogen phosphate, 1.0; Potassium chloride, 0.5; magnesium sulphate, 0.2; Calcium chloride, 0.1; yeast extract, 0.5; Agar, 20 and Cellulose, 10. After 7 days incubation at 28°C, plates were flooded with chloro-iodide of zinc. The uncoloured zone gave a measure of the cellulolytic power of the fungi.

Isolates were screened on MP-7 and MP-5 media of Hankin et al. (1971) for pectin lyase (PL) and polygalacturonase (PG), respectively. After growth of organisms for 7 days at 28°C. Pectolytic activities on both media were determined by flooding plates with 7 mol/L HCl solution. This precipitate intact pectin. Pectolytic moulds were thus surrounded by clear zones against ■ opaque medium. The extent of zone of clearing around moulds was used as ■ measure of the degree of pectolytic activity.

Mycotoxin-producing potential of the isolated fungi. Fifteen isolates of both *Alternaria alternata* and *Aspergillus flavus*, 17 isolates of *A. niger* and 2 isolates of

each of *A. fumigatus*, *Cochliobolus spicifer* and *Penicillium italicum* were screened for their ability to produce mycotoxins on yeast extract sucrose medium (YES). Erlenmeyer flasks (250 ml), each containing 50 ml of YES medium were inoculated by the examined fungi and were incubated as stationary cultures at 28°C for 10 days. Two replicates of each isolate were analyzed.

At the end of the incubation period, the contents of each flask were homogenized with 50 ml chloroform for 5 min in a high speed blender (18000 g). Extraction was repeated three times. The combined chloroform extract was washed with distilled water, dried over anhydrous sodium sulphate, filtered and dried to near dryness on a rotary evaporator. The residue was diluted with chloroform to one ml. The chloroform solution was analysed for the presence of different mycotoxins using thin-layer chromatography as described by Gimeno (1979). With *A. fumigatus*, the extract was analysed for the presence of gliotoxin by spraying plates with silver nitrate solution (Taylor, 1967). In case of *Alternaria*, liquid cultures were extracted twice with ethyl acetate (30 ml) by overnight shaking at dark and filtration. The two extracts were combined, dried (anhydr- ous sod. sulphate), evaporated under reduced pressure, and the residue dissolved in l ml of methylene chloride for TLC analysis.

TLC analysis was performed on precoated silica gel plates of Kieselgel G type 60 (MERCK) by using (chloroform: acetone) (88:12) and (toluene:ethylacetate:90% formic acid) (60:30:10) as solvent systems. Alternariol (AOH) and alternariol monomethyl ether (AME) were visualized under long wave length UV light with their characteristic fluorescence (Visconti et al., 1986). Standards of AOH and AME were purchased from Sigma Chemical Comp., U.S.A.

Mycotoxin production in infected healthy lemons. The toxigenic isolates of both *Alternaria alternata* and *Aspergillus flavus* which produced mycotoxins in YES medium were inoculated into the surface of healthy lemons. The inoculation was performed by placing a square block of Czapek's dextrose agar medium with the fungal spores in a window-shaped wound on lemon (Vinas et al., 1992). Inoculated fruits were placed in plastic bags with one end left open and incubated at 28 ± 1°C for 15 days (Stinson et al., 1981).

Extraction procedures and TLC analysis. The decayed tissue was cut from infected fruits and homogonized at top speed in the warring blender for 2 min. In case of *Alternaria*, the homogenized tissue was adjusted to pH 2.0 by adding 2 N HCl. A certain volume of (chloroform: ethanol) (4:1) (v/v), about twice the weight of the fruit was added. The mixture was again homogenized for 2 min. These mixtures were centrifuged for 10 min at 2500 g to break the emulsion and to separate the mixture into distinct layers (Stinson et al., 1981). The organic phase containing the toxins was dried over anhydrous sodium sulphate, filtered, evaporated and redissolved in 2 ml of methanol for TLC analysis according to Gimeno (1979) for aflatoxins and Visconti et al. (1986) for *Alternaria* toxins.

RESULTS AND DISCUSSION

Results presented in Table I revealed that 9 species related to 7 genera were recovered from rotted lemon fruits. It was obvious that *Alternaria alternata* was the

main causer of lemon rot followed by *Aspergillus flavus* and *A. niger*. The numbers of cases of isolation of these fungi were high. These results are in agreement with those of Singh (1980) and Stinson et al. (1981) who published that citrus fruits such as lemons are affected by *Alternaria* rot. In a similar study, Vinas et al. (1992) found that 81.9% of 110 strains of *Alternaria* isolated from 157 decayed apples belonged to the species *A. alternata*.

Genera and species	No. of isolates
Alternaria alternata (Fr.) Keissler	36
Aspergillus flavus Link	31
A. niger Tan Tieghem	26
A. fumigatus Fresenius	3
Ulocladium atrum Preuss	5
Cochliobolus spicifer Nelson	5
Penicillium italicum Wehmer	4
Mucor hiemalis Wehmer	3
Acremonium strictum W.Gams	2

Table I: Number of cases of isolation (out of 50 samples) of fungal genera and species recovered from rotted lemon fruits.
Tableau I: Nombre d'isolements (50 essais) des différentes espèces sur citrons moisis.

In Nigeria, Fawole & Odunfa (1992) found that the aspergilli, of which *A. flavus* and *A. niger* were the most dominant, formed the largest group of fungi causing decay of citrus fruits.

The other identified fungi were *A. fumigatus, Cochliobolus spicifer, Ulocladium atrum, Penicillium italicum, Mucor hiemalis* , and *Acremonium strictum*. Many of these fungi were previously isolated from rotted citrus and other fruits (Kursanoff & Alexeyeva, 1938; Thompson & Eribo, 1984; Fawole & Odunfa, 1992).

The ability of 37 fungi isolated from rotted lemons, to produce enzymes on solid media is shown in Table 2. The term "production" means here both synthesis of the enzyme by the fungus and its activity in the medium after it is "produced". From the data, it was apparent that the tested fungi were more able to produce cellulases than pectinases (PL and PG).

Results also clearly show that not only all the tested fungi were able to produce cellulase but also most of them were good producers of this enzyme. Among test fungi, *A. flavus* exhibited the highest ability in cellulase production followed by *A. niger*. It is worthmentioning that the enzymatic ability differs from fungal species to another and even between isolates of the same species. These results are in agreement with those of

Moubasher & Mazen (1991) who published that most of fungal isolates related to 92 species proved to be active to utilize cellulase but with different degrees. Similar results were obtained by different authors (Stewart & Walsh, 1972; Gomes et al., 1989).

Data in Table II indicate that the production of pectolytic enzymes was verified by many of the examined fungi. This could be attributed to the fact that pectic

Organisms	No. of tested isolates	Cellulase		Pectin lyase (PL)		Polygalacturonase (PG)	
		N*	Activity	N	Activity	N	Activity
Acremonium strictum	1	1	+	1	−	1	−
Alternaria alternata	10	4	+++	4	+++	2	++++
		5	++	4	++	3	++
		1	+	1	+	4	+
				1	−	1	−
Aspergillus flavus	10	4	++++	4	+++	1	+++
		2	+++	4	++	4	++
		2	++	1	+	4	+
		2	+	1	−	1	−
A. fumigatus	2	1	++++	1	++++	2	++
		1	+++	1	++		
A. niger	7	2	++++	2	++++	1	++
		3	+++	1	+++	5	+
		1	++	3	+	1	−
		1	+	1	−		
Cochliobolus spicifer	2	2	+++	2	++	1	+
						1	−
Mucor hiemalis	1	1	+	1	+	1	−
Penicillium italicum	2	1	+++	1	++	1	+
		1	−	1	−	1	−
Ulocladium atrum	2	2	+++	2	++	1	++
						1	−

* . Number of isolates
\+ . low activity
++ . fair activity
+++ . high activity
++++ . very high activity
− . no activity

Table II: Enzymatic activities of some fungi isolated from rotted lemon.
Tableau II: Activité enzymatique des espèces fongiques isolées de citrons moisis.

substances are known to enhance pectic enzyme production (Fogarty & Kelly, 1983). In the present investigation, some isolates related to *Aspergillus* proved to be good producers of pectinases. Many species of *Aspergillus* are known to be pectolytic and are

in fact the usual source of industrial pectinases (Blain, 1975; Barbesgaard, 1977; Fawole & Odunfa, 1992).

One isolate of each of *Acremonium strictum*, *Alternaria alternata*, *A. flavus*, *A. niger* and *P. italicum* did not show any pectolytic activity during this study (Table II).

During screening for mycotoxigenicity of fungi isolated from rotted lemons, 17 out of 43 isolates were toxigenic (Table III). The toxigenic isolates were related to *Alternaria alternata* (9 isolates) and *A. flavus* (8 isolates). With the remaining tested fungi namely *A. fumigatus*, *A. niger*, *Cochliobolus spicifer* and *P. italicum*.

Organism	No. of tested isolates	No. of toxigenic isolates	Mycotoxins detected
Alternaria alternata	15	9	Alternariol (AOH) & Alternariol monomethyl ether (AME)
Aspergillus flavus	15	■	Aflatoxins B_1 & B_2
A. fumigatus	2	-	-
A. niger	7	-	-
Cochliobolus spicifer	2	-	-
Penicillium italicum	2	-	-

Table III: Mycotoxin-producing potential of some fungi isolated from rotted lemons.
Tableau III - Mycotoxines produites par les espèces fongiques isolées de citrons moisis.

Alternaria alternata was the main mycotoxin producer where 60% of its isolates produced toxins. 2 isolates produced alternariol (AOH) and one isolate produced alternariol monomethyl ethers [AME] whereas 6 isolates produced both AOH and AME. These results are in accordance with those of Vinas et al. (1992) who reported that *Alternaria* isolated from decayed apples produced AOH and AME on yeast extract sucrose medium. They found that among the examined isolates, 14.7% produced AOH and 22.6% produced AME.

From 15 isolates of *A. flavus*, 18 isolates produced aflatoxins B and ■ . 2 isolates produced aflatoxin B and 6 isolates produced aflatoxins ■ and B. Our data are in agreement with those of Varma & Verma (1987) who found that 3 isolates of *A. flavus* out of 7 isolates from oranges were aflatoxigenic. The association of aflatoxigenic species such as *A. flavus* and *A. parasiticus* with citrus fruits was previously reported (Nolte & Vanloeseeke, 1940;Varma, 1985).

The ability of toxigenic isolates of both *Alternaria alternata* and *A. flavus* to produce mycotoxins in healthy lemons were shown in Table IV. From 9 isolates of *Alternaria alternata*, 16 isolates produced AOH and AME. These findings are in good agreement with those of Stinson et al. (1981) who found that AOH and AME were the predominant mycotoxins which were produced in *Alternaria*- infected oranges and lemons. These toxins were also the major mycotoxins produced in apple and other fruits infected with *Alternaria* spp. (Stinson et al., 1980).

Toxigenic fungus	No. of tested isolates	No. of + ve isolates	Mycotoxins detected
Alternaria alternata	9	6	Alternariol (AOH) & Alternariol monomethyl ether (AME)
Aspergillus flavus	8	3	Aflatoxins B_1 & B_2

Table IV: Ability of toxigenic fungi to produce mycotoxins on healty lemons.
Tableau IV: Capacité des espèces fongiques isolées à produire des mycotoxines sur citrons sains.

Three isolates (37.5%) of toxigenic *A. flavus* produced aflatoxins B and B in lemons. The experimental production of aflatoxins in citrus fruits and fruit juice such as mosambi (*Citrus sinensis*), orange (*C. reticulata*) and lemon (*C.limon* Syn.) have been reported by other workers (Singh & Sinha, 1982; Varma, 1985).

The comparatively lower yield of aflatoxins by toxigenic isolates in lemons than the yeast extract sucrose liquid medium may be due to the presence of citric acid and citrus oil (Alderman & Marth, 1976). Keffard (1966) reported the presence of acids (5-6%) in lemon with the dominance of citric acid. Citric acid supports growth but not aflatoxin production (Davis & Diener, 1968).

Results obtained during this study clearly indicated that fungi isolated from rotted lemons had a high ability to produce cell-wall degrading enzymes which are important in breakdown of organic matter and in fruits deterioration. In addition, the production of some mycotoxins in infected lemons may constitute a potential hazard to human health. The possibility exists that contaminated fruits may be incorporated into processed products, such as juice and preserves, through faulty sorting procedures or neglect and thus constitute a potential health hazard.

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